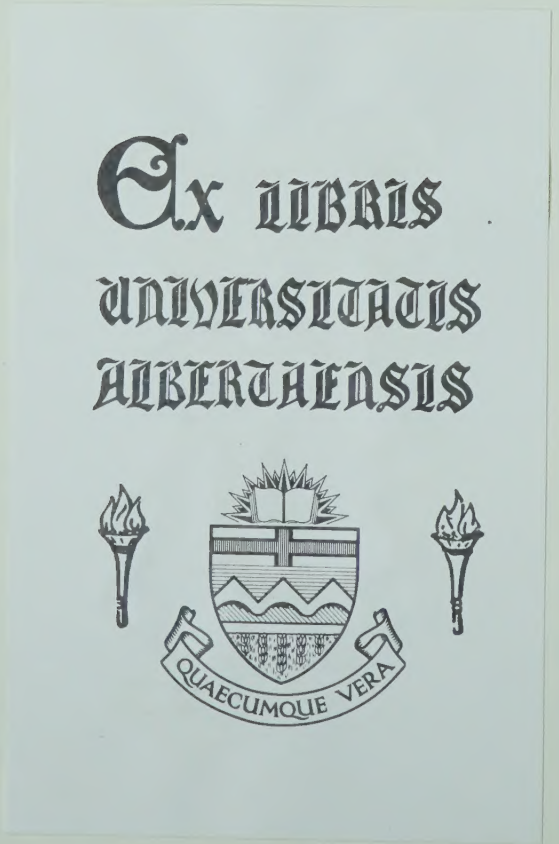

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SEPARATION AND CHARACTERIZATION OF ACYL LIPIDS OF BEAN CHLOROPLASTS

by

© GLENN MAURICE PULLISHY

A THESIS

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "Separation and Characterization of Acyl Lipids of Bean Chloroplasts" submitted by Glenn Maurice Pullishy in partial fulfilment of the requirements for the degree of Master of Science.

ABSTRACT

Chloroplasts were isolated from the leaves of 12 day old Kinghorn wax beans (Phaseolus vulgaris) with a sucrose-phosphate buffer and purified on a discontinuous sucrose density gradient. The lipids were extracted and purified on a Sephadex LH-20 column. The purified lipid was separated into four fractions by a silica gel column chromatographic method. The first two fractions contained most of the pigments while fraction 3 contained the monogalactosyl diglyceride and a phospholipid. Fraction 4 contained the digalactosyl diglyceride and sulphoquinovosyl diglyceride. The glycolipids were separated further by silica gel HR thin-layer chromatography. The glycolipid concentrations were obtained by estimating the sugar components spectrophotometrically after they had been hydrolized on the adsorbent in 2 N sulfuric acid and an aliquot was reacted with phenol and sulfuric acid.

The lipids extracted from these chloroplasts contained only four major acyl lipids. These were monogalactosyl diglyceride, digalactosyl diglyceride, sulphoquinovosyl diglyceride and phosphatidyl glycerol. Linolenic acid was almost the exclusive fatty acid of monogalactosyl diglyceride and represented more than 75% of the fatty acids of digalactosyl diglyceride. In the sulpholipid, less than half of the fatty acid was linolenic acid. The remainder of the fatty acids in these lipids was predominantly palmitic acid.

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INTRODUCTION

Recent years have seen major advances in our knowledge of the lipid chemistry and biochemistry of photosynthetic tissues, but most of the work has been performed on intact leaves rather than on isolated chloroplasts. This is largely a reflection of the difficulty experienced in isolating pure whole chloroplasts without loss or alteration of their components and with retention of their full metabolic capacities. Of the work done on isolated chloroplasts, only three investigations have dealt with quantitative analyses of the glycolipids (Wintermans, 1960; Allen, Good, Davis, Chisum and Fowler, 1966; Ongun, Thomson and Mudd, 1968).

In the past, the main limitations were the methods available for separation of the lipid components for quantitative analyses. Earlier methods using silicic acid columns gave partial resolution of the major lipids and contamination with chlorophyll was a serious problem. Other methods, such as silicic acid impregnated paper and silicic acid thin-layer chromatograms were subject to oxidation of the lipids during separation.

Most of the methods involving column chromatography require that non-lipid impurities first be removed from the lipid extracts. Frequently this is achieved by repeated washing of the extract with water or various salt solutions. This results in large losses of carbohydrate containing lipids. A method suitable for removing the non-lipids without washing, developed by Maxwell and Williams (1967), makes use of a Sephadex LH-20 column. More recently they (Maxwell and

Williams, 1969) reported that a short silicic acid column was better than previous methods in removing chlorophyll contamination, and Roughan and Batt (1968) have developed a rapid method for estimating very small amounts of sulpholipid and galactolipids from thin-layer chromatograms.

Accurate analyses of the acyl lipid composition of chloroplasts are desirable not only because of the significant role of lipids in membrane structure and in photosynthesis, but also because lipids represent more than one third of the chloroplast components. This investigation was designed to adapt existing methods to make them more suitable for quantitative estimations of glycolipids from isolated chloroplasts. In addition, it reports the details of isolation and characterization of the major acyl lipids of bean chloroplasts.

LITERATURE REVIEW

Leaf Lipids

The lipids of leaves are known to consist of many components. These include neutral lipids such as hydrocarbons, waxes, alcohols, quinones, sterols, sterol esters and glycerides (Zill and Harmon, 1962; Weenink, 1962; Nichols, 1963; Lepage, 1964; Sastry and Kates, 1964a; Sastry and Kates, 1964b). Phospholipids such as phosphatidyl glycerol, polyglycerol phosphatide, phosphatidyl inositol, phosphatidyl choline, phosphatidyl ethanolamine and normally traces of phosphatidyl serine, phosphatidic acid and phytoglycolipid are also found in plant leaf tissue (Benson and Maruo, 1958; Benson and Stickland, 1960; Wintermans, 1960; Kates, 1960; Lepage, Mumma and Benson, 1960; Carter, Galanos, Hendrickson, Nakayama, Nakazawa and Nichols, 1962; Nichols, 1963; Roughan and Batt, 1969). A sulpholipid has also been found in all plant leaves examined (Benson, Daniel and Wiser, 1959; Benson, 1963; O'Brien and Benson, 1964; Maxwell and Williams, 1968; Roughan and Batt, 1968; Roughan and Batt, 1969). Quantitatively the most important lipids of the leaf where found to be the galactosyl diglycerides, monogalactosyl diglyceride and digalactosyl diglyceride (Benson and Maruo, 1958; Benson, Wintermans and Wiser, 1959; Wintermans, 1960; Kates, 1960; Zill and Harmon, 1962; Allen, Good, Davis and Fowler, 1964; Sastry and Kates, 1963; Hirayama, 1965; Maxwell and Williams, 1968; Roughan and Batt, 1968; Roughan and Batt, 1969). The major lipids of the leaf have been found to be concentrated in the chloroplasts.

Chloroplast Lipids

The total lipid content of spinach chloroplasts was found by Comar (1942) to make up 37 per cent of the dry weight. Park and Pon (1963) later reported that lipids make up 52 per cent of spinach chloroplast lamellae.

There are fewer kinds of lipid components in leaf chloroplasts than in whole leaves. Starting with the minor lipids, chloroplasts contain several different kinds of quinone (Henninger and Crane, 1963; Henninger, Barr, Wood and Crane, 1965). Together the quinones constitute about 3 per cent by weight of the total chloroplast lipid. Menke and Jacob (1942) reported that 1.8 to 2.5 per cent of the lipids of spinach chloroplasts consisted of sterols. Zill and Harmon (1962) also claim to have found sterols in spinach chloroplasts, but no quantitative data were given. Nichols (1963) was unable to find any sterol in lettuce chloroplasts. Recently Mercer and Treharne (1965) reported the presence of small amounts of sterol in Phaseolus chloroplasts and tentatively identified most of it as being cholesterol. The presence of sterol esters as well as sterols in chloroplasts has been reported for Sapium sebiferum (Hirayama, 1965) and Beta vulgaris (Baily, Thornber and Whyborn, 1966). Stevenson, Hemming and Morton (1963) have shown that tobacco chloroplasts contain isoprenoid alcohols and recently Wellburn and Hemming (1965) have found isoprenoid alcohols make up about 0.5 per cent of the total lipids of horsechestnut leaf chloroplasts. About 82 per cent of the chloroplast lipids are known components. The unidentified 18 per cent probably consist of small amounts of minor constituents such as free fatty acids, precursors of

phytol, chlorophyll and carotenoids and their breakdown products (Lichtenthaler and Park, 1963).

The chloroplast contains only four acyl lipids in major proportions. These major chloroplast lipids are the three glycolipids, monogalactosyl diglyceride, digalactosyl diglyceride and sulphoquinovosyl diglyceride and the phospholipid, phosphatidyl glycerol (Benson and Maruo, 1958; Benson, Wintermans and Wiser, 1959; Wintermans, 1960; Nichols, 1963; Allen, Good, Davis, Chisum and Fowler, 1966; Ongun, Thomson and Mudd, 1968). The two galactosyl diglycerides are the most abundant lipid of the chloroplast. Between them they constitute about 40 per cent by weight of the total lipid (Park and Biggins, 1964).

A. Galactosyl Diglycerides

Carter and co-workers (1956) were the first to isolate and identify monogalactosyl diglyceride and digalactosyl diglyceride. They isolated the two galactosyl diglycerides from wheat flour and established their chemical structures as 2,3-di-O-acyl-1-O-(β-D-galactopyranosyl)-D-glycerol (monogalactosyl diglyceride) and 2,3-di-O-acyl-1-O-(6-O-α-D-galactopyranosyl-β-D-galactopyranosyl)-D-glycerol (digalactosyl diglyceride). Miyana and Benson (1963) later showed by quite different techniques that mono- and digalactosyl diglyceride of Chlorella had the same structure. Sastry and Kates (1963) also isolated and characterized these same two lipids from runner bean leaves. Wintermans (1960) separated the lipids of leaves and isolated chloroplasts of beet leaves by two dimensional paper

chromatography. He measured the concentrations of the galactosyl diglyceride in leaves and chloroplasts and found that they were specific to the chloroplasts. He reported that the concentration of monogalactosyl diglyceride in the chloroplasts was 24 X 10^4 moles per litre as compared to 13 X 10^4 moles per litre of digalactosyl diglyceride. His results also indicated that galactosyl diglycerides make up about 41 per cent of the major acyl lipids of the beet chloroplast. In his experiments with Sambucus and Phaseolus, Wintermans found that the concentrations of galactosyl diglycerides in the green leaves were significantly higher than in the yellow ones. The yellow leaves were given day lengths of only 0.5 to 2 hours as opposed to 8 hours for the green leaves. Zill and Harmon (1962) used silicic acid chromatography to separate the lipids of whole spinach leaves and of the chloroplasts. They found that the galactosyl diglycerides were the major lipids of spinach chloroplasts. Because of poor separations and the interference with chlorophyll they were not able to report these on a quantitative basis. Nichols (1963) used thin-layer chromatography to separate the lipids of lettuce leaves and chloroplasts and found that the galactosyl diglycerides of the leaf were located in the chloroplast and these were the most abundant lipids of the cell. Allen and co-workers (Allen, Good, Davis, Chisum and Fowler, 1966) separated the lipids of spinach chloroplast lamellae with diethylaminoethyl (DEAE) cellulose column chromatography. They showed that monogalactosyl diglyceride and digalactosyl diglyceride were confined to the chloroplast lamellae. They also tentatively identified a trigalactosyl diglyceride in the chloroplast of spinach. Benson and his

co-workers (1958), earlier reported the synthesis of radioactive trigalactosyl diglyceride along with other lipids by exposing Chlorella cells to radioactive carbon dioxide for a short period of time under photosynthetic conditions. Ongun and Mudd (1968) reported that they never observed the presence of trigalactosyl diglyceride if the lipids were extracted immediately after the isolation of the chloroplasts. However they found that radioactive trigalactosyl diglyceride was formed during the incubation of chloroplasts with uridine diphosphate galactose-^{14}C. They suggested that this may possibly indicate that the mechanism responsible for the control of galactolipid synthesis was somewhat altered by removing the chloroplasts from their natural environment in the cell. Webster and Chang (1969) have recently isolated two polygalactolipids from spinach chloroplasts which they designated as components A and B. They also obtained these two polygalactosyl diglycerides from glycolipid products synthesized with spinach chloroplast enzymes using uridine diphosphate galactose-^{14}C as a galactose donor. They purified these lipids by silicic acid column chromatography and thin-layer chromatography. One of these lipids, component A, was identified as trigalactosyl diglyceride and component B was tentatively identified as tetragalactosyl diglyceride. The relative amounts as molar ratio of galactolipids in spinach chloroplasts was reported as monogalactosyl diglyceride 60, digalactosyl diglyceride 30, trigalactosyl diglyceride 5, tetragalactosyl diglyceride 1.

B. Sulphoquinovosyl Diglyceride

Plant sulpholipid was discovered by Benson, Daniel and Wiser in 1959. Their laboratory (Lepage, Daniel and Benson, 1961) later characterized it as being a glyceride of 6-sulphoquinovosyl glycerol. It has since been found in all photosynthetic plants in which it has been sought. Roughan and Batt (1969) recently reported its exisistence in twenty photosynthetic tissues ranging from the unicellular green alga Mesotaenium caldariarum to the leaves of higher plants as well as in parsnip root cortex. Wintermans (1960) measured the concentration of sulpholipid in the leaves and chloroplasts of beets. He found that the sulpholipid was less specifically connected with the photosynthetic apparatus than the galactosyl diglycerides. The concentration of sulpholipid in the chloroplast was found to be 3×10^{4} moles per litre as compared to 9×10^{4} moles per litre found in the total leaf. He also found from experiments with green vs. yellow leaves that the concentrations of sulpholipid were not significantly different at different chlorophyll concentrations. Roughan and Batt (1969) have indicated that sulpholipid levels in red clover leaves may be reduced three-fold in the early morning as compared with later in the day. Zill and Harmon (1962) found that sulpholipid was mainly in the chloroplasts of spinach leaves. Sulpholipid has recently been shown to be specifically located in the lamellar part of the chloroplast of spinach (Shibuya and Maruo, 1965; Allen, Good, Davis, Chisum and Fowler, 1966).

C. Phosphatidyl Glycerol

Phosphatidyl glycerol was discovered by Benson and Maruo (1957). Haverkate and co-workers (Haverkate, Houtsmuller and van Deenen, 1962; Haverkate and van Deenen, 1956) found it was cleaved by phospholipases, the products confirmed the structure proposed by Benson as 1, 2-diacylglycerol-3phosphoryl-1-glycerol. Benson, Wintermans and Wiser (1959) reported the presence of phosphatidyl glycerol in spinach chloroplasts. Wintermans (1960) later reported the presence of four phospholipids in the whole leaves and the chloroplasts of spinach and beet. He found that phosphatidyl glycerol was concentrated in the chloroplast whereas phosphatidyl choline, phosphatidyl inositol and phosphatidyl ethanolamine were found mainly in the chlorplasts. Allen and co-workers (Allen, Harayama and Good, 1966) found that phosphatidyl glycerol made up 16 per cent of the total acyl lipids of spinach chloroplast lamellae. They also found small amounts of phosphatidyl choline and phosphatidyl inositol in the lamellae. Ongun, Thomson and Mudd (1968) have recently reported that chloroplasts isolated from tobacco leaves contained 74 per cent of the cellular phosphatidyl glycerol. They also found that phosphatidyl inositol, phosphatidyl choline and phosphatidyl ethanolamine are mainly in the cytoplasmic fraction but are also found in the chloroplast fraction. The occurrence of phosphatidyl choline, phosphatidyl inositol and phosphatidyl ethanolamine in chloroplasts is debatable and the uncertainty arises from the difficulty experienced in isolating uncontaminated chloroplasts from leaves using aqueous media. A study of a variety of chloroplast preparations shows that the purer

the preparation, the smaller the proportion of phosphatidyl choline in the lipid extract (Nichols and James, 1968). On the other hand, no chloroplast preparations have yet been reported that do not contain phosphatidyl choline.

Fatty Acids

The total fatty acid composition of the leaves of higher plants show a consistent pattern in which only slight quantitative variations occur between plant classes. The major fatty acids in leaves, on a quantitative basis are linolenic (9, 12, 15-octadecatrienoic acid), linoleic acid (9, 12-octadecadienoic acid) and palmitic acid (hexadecanoic acid), and in some plant leaves such as spinach and tobacco, 7, 10, 13-hexadecatrienoic acid has been found to be a major fatty acid (Heyes and Shorland, 1951; Debuch, 1961). The monoenoic acid, trans-3-hexadecenoic acid is a minor component of the total fatty acids but has been found only in the green photosynthetic tissue of plants. The fatty acid is also unique in that it has a trans configuration instead of the usual cis (Weenink and Shorland, 1964; Allen, Good, Davis and Fowler, 1964; Nichols, 1965a; Nichols, Stubbs and James, 1967). This trans-3-hexadecenoic acid is absent from etiolated tissues (Nichols, Wood and James, 1965; Nichols, 1965b).

Chloroplasts contain large amounts of fatty acids combined in various forms and possibly free as well. Crombie (1958) found that fatty acids (free and combined) made up about 44 per cent by weight of the total lipids of the chloroplasts of Vicia faba.

The lipids of leaf chloroplasts contain a higher proportion of polyenoic acids, particularly linolenic acid than do those from

other parts of the leaf (James and Nichols, 1966; Wolf, 1961). The fatty acids of chloroplasts are not randomly distributed between the different acyl lipids but show a very high degree of specificity for certain lipids (Allen, Hirayama and Good, 1966; Nichols, 1965b). The only information regarding the fatty acid composition of the individual lipids from isolated chloroplasts is from the work done by Allen and co-workers on spinach (Allen, Hirayama and Good, 1966; Allen, Good Davis Chisum and Fowler, 1966). On the other hand, there is a lot of data concerning the composition of the individual lipids from the unfractionated leaf tissue.

Linolenic acid which is the most abundant fatty acid is primarily concentrated in the two galactosyl diglycerides, especially in the monogalactosyl diglyceride. Digalactosyl diglyceride also contains significant quantities of palmitic acid (Nichols, Harris and James, 1965; Allen, Hirayama and Good, 1966; Nichols, 1965b; Rosenberg, Gouaux and Milch, 1966; Rosenberg and Gouaux, 1967; Allen, Good, Davis and Fowler, 1964; Nichols, 1965a; Nichols, Stubbs and James, 1967; O'Brien and Benson, 1964; Gardner, 1968). The fatty acid composition of sulphoquinovosyl diglyceride and phosphatidyl glycerol is predominately palmitic and linolenic acids (Nichols, Harris and James, 1965; Allen, Hirayama and Good, 1966; Nichols, 1965b; Allen, Good, Davis and Fowler, 1964; Nichols, 1965a; O'Brien and Benson, 1964; Nichols, Stubbs and James, 1967). Phosphatidyl glycerol also contains all the trans-3-hexadecenoic acid present in the leaf (Haverkate and van Deenen, 1965; Allen, Good, Davis and Fowler, 1964; Weenik, 1964; Nichols, 1965a; Allen, Good, Davis,

Chisum and Fowler, 1966). The positional distribution of the fatty acids of phophatidyl glycerol were investigated after complete breakdown by the action of phospholipase A from snake venom, which liberates specifically the fatty acids from the 2-position of the lipid. Analysis by Haverkate and van Deenen (1965) showed that the trans-3-hexadecenoic acid was located exclusively at the 2-position, while a predominant part of the linolenic acid appeared to occupy the 1-position. Phosphatidyl glycerol is not a specific lipid of photosynthetic tissues in so far as this phospholipid was detected in relatively large amounts in many non-photosynthetic bacteria, and it was found to be a minor lipid component of animal tissues. On the other hand, the chemical make up of the phosphatidyl glycerol from various sources has been found to be quite different. A specific association between phosphatidyl glycerol and trans-3-hexadecenoic acid appears to occur only in those organisms in which photosynthesis is of the type found in green plants. This combination has not been found so far in this lipid from animal tissues, non-photosynthetic bacteria or from photosynthetic bacteria (van Deenen and Haverkate, 1966).

Fatty Acid Composition Changes

Crombie and co-workers were among the earliest to investigate the effects of light and darkness upon the fatty acid composition of plant tissues (Crombie and Comber, 1956; Hardman and Crombie, 1958; Crombie, 1958). These workers noted marked increases in the linolenic acid content of watermelon cotyledons during greening.

Comparing light and dark-grown cultures of Euglena gracilis (Erwin and Bloch, 1962; Erwin and Bloch, 1963; Rosenberg, 1963; Hulanicka et al., 1964), large amounts of linolenic acid were found in light-grown cells. Newman (1962) and Wallace and Newman (1965) using bush beans showed that the chloroplasts of light-grown material contain a higher ratio of unsaturated to saturated fatty acids, and a higher ratio of C_{18} to C_{16} fatty acids than do the proplastids of dark-grown material. Wolf, Coniglio and Bridges (1966) analized the fatty acids of the chloroplasts of wheat grown either in continuous darkness or under a 14-hour photoperiod. They found a pronounced increase in linolenic acid in the light, with corresponding relative decreases in palmitic, stearic and oleic acids. Newman and Wallace (1965) showed that decreased rates of photosynthesis lead to decreases in the concentration of linolenic acid and galactolipids of leaf tissue. Gray, Rumsby and Hawke (1967) have recently shown a relationship between the levels of chlorophyll and unsaturated fatty acids in green tissue of grasses. As the chlorophyll level rose, the linolenic acid composition increased. They reported that there were relatively small changes in the fatty acid composition of monogalactosyl diglyceride of grasses of different chlorophyll content. They concluded that the observed increase in linolenic acid content associated with increased photosynthetic activity is due to an increase in the total galactolipid content of the tissue and not to changes in the constituent fatty acid components of monogalactosyl diglyceride. Nichols, Stubbs and James (1967) demonstrated that the increase in linolenic acid content observed upon illumination of dark-grown

Ricinus seedlings occurred primarily in the galactolipids. They also demonstrated that of these galactolipids, only monogalactosyl diglyceride and sulphonquinovosyl diglyceride were enriched with this acid. Appelqvist, Boynton, Stumpf and Wettstein (1968) also demonstrated the enrichment of linolenic acid in monogalactosyl diglyceride and sulphoquinovosyl diglyceride of greening barley. They, however, did not find an enrichment of this fatty acid in the digalactosyl diglyceride fraction. In these experiments, the increase in linolenic acid content of the total lipids and the enrichment of the monogalactosyl diglyceride fraction were found to occur in the first 15 hours of greening, with little change thereafter. This coincided with chlorophyll synthesis and the formation of the chloroplast lamellar system.

Changes in composition of fatty acids of leaves has been observed with the age of the plant. Younger leaves appear to contain more unsaturated fatty acids and older ones more saturated acid. Hawke (1963) has studied the difference in the composition of the lipids of new growth and mature ryegrass. The fatty acids of the new growth were linolenic (75%), palmitic (12%) and linoleic (8.4%). In the mature grass the composition was linolenic (65%), palmitic (16%) and linoleic (12%). It was also found that young leaves of the squash plant had a lower ratio of saturated to unsaturated fatty acids than older leaves (Wallace and Newman, 1965). Newman also found that the fatty acid composition of the leaves from the different nodes of bush beans varied, the younger leaves contained more C_{18} unsaturated acids and older ones more palmitic acid. He also reported that plastids

from mature tissue of bush bean contained less chlorophyll and have a higher saturated to unsaturated fatty acid ratio than plastids from young tissue. Klopfenstein and Shigley (1967) showed that in the sulpholipid of alfalfa leaves, the proportion of palmitic acid to linolenic acid increased during aging of the plant. It has also been suggested (Roughan and Batt, 1969) that sulpholipid levels in the early morning may be reduced three-fold compared with later in the day. If it is a general property of plants, that as they mature their leaf lipids become more saturated, and if the lipid composition varies depending on the time of day of harvest, it will not be possible to assign a characteristic fatty acid composition to a plant lipid on the basis of only one analysis.

Function of Lipids in Chloroplasts

The structure of lipids present in the chloroplast are now fairly well understood, but their exact location and function have yet to be clearly defined. These lipids could function as either chemical or structural components of the chloroplast and could also serve both functions.

A. Metabolic Function

Several possibilities have been considered for the chemical involvement of the acyl lipids in the various metabolic processes carried out within the chloroplast. One of the earlier suggestions was made by Benson, Wintermans and Wiser (1959) who found that the most abundant lipids of the chloroplast were galactosyl diglycerides. From this they suggested that the function of these lipids was as a

reservoir of carbohydrate within the chloroplast. They also mentioned that these lipids may participate in carbohydrate metabolism as well. Ferrari and Benson (1961) studied the rates of ^{14}C incorporation into lipids and their components during steady state photosynthesis with $^{14}CO_2$ by Chlorella pyrenoidosa. They found that the fatty acids of monogalactosyl diglyceride and phosphatidyl glycerol were rapidly labelled. The glycolipids were labelled in the order of monogalactosyl diglyceride, digalactosyl diglyceride and sulphoquinovosyl diglyceride. They also observed a rapid turnover of label in the sugar moieties of these three glycolipids. The turnover of label in the sugar portion of the digalactosyl diglyceride and the sulpholipid was faster than the turnover of labled fatty acids of these two glycolipids. From these results they concluded that the galactosyl diglyceride and possibly the sulpholipid are involved in sugar metabolism and transport in the chloroplast.

It has been suggested by Erwin, Hulanicka and Bloch (1964) and supported by others (Kates and Volcani, 1966; Patton, Fuller, Loebinch and Benson, 1966; Appleman, Fulco and Sugarman, 1966), that the high content of linolenic acid in the galactolipids, which occur almost exclusively in the chloroplast, may be related to a functional role of these lipids in photosynthesis. These authors suggested that linolenic acid may be chemically involved in electron transport or is a necessary physical component of the oxygen-evolving reaction in photosynthesis. On the other hand oxygen may be required for the photosynthesis of linolenic acid from oleic acid, and that the increased synthesis of this fatty acid after photosynthesis begins may

be merely a reflection of the requirement for oxygen (O'Brien and Benson, 1964). The observation by Holton and co-workers (1964) as well as Nichols and Wood (1968) and Parker et al. (1967) that some blue-green algae can carry out the Hill reaction despite the lack of polyunsaturated fatty acids indicates that the proposal by Erwin et al. (1964) could not apply universally.

It has been pointed out earlier in the review that in all photosynthetic tissue of higher plants studied, trans-3-hexadeconic acid is specifically located on phosphatidyl glycerol which is the major phospholipid of chloroplasts. Moreover, it was absent from all etiolated photosynthetic tissue which has been examined (Nichols, 1965b; Nichols, Wood and James, 1965; Appelqvist, Boynton, Stumpf and Wettstein, 1968). From these observations, Nichols and his co-workers (Nichols, Wood and James, 1965) suggested that this acid might have some specific active role in photosynthesis. Their suggestion may be supported by the fact that the fatty acids of phosphatidyl glycerol turn over more rapidly than those of any other lipid in the chloroplast (Ferrari and Benson, 1961; Nichols, Stubbs and James, 1967; Nichols and James, 1967). On the other hand, it was shown by Nichols (1965b) that this fatty acid is not present in some blue-green algae. From this, one can conclude that unless there is a difference in the mechanisms of photosynthesis in blue-green algae and that of leaves and other algae, this fatty acid cannot be essential for photosynthesis in all species.

B. Structural Function

Recently Weier and Benson (1967), Muhlethaler (1966) and Bamberger and Park (1966) suggested how the acyl lipids and other components of the chloroplast lamellae could be arranged. Weier and Benson (1967) pictured the whole chloroplast lamellae consisting of mixed protein and lipid components. They suggested that the molecules of protein are relatively hydrophobic in nature and that the hydrocarbon chains of the chlorophyll, carotenoids and the lipids are buried within the coiled protein molecular band in hydrophobic association. On the other hand, Kreuty (1966) had suggested that the lipid and protein components are separate.

Muhlethaler (1966) and Park and his co-workers (Bramberger and Park, 1966; Branton and Park, 1967) using the technique of freeze-etching found particles of two size categories in the chloroplast lamallae. Muhlethaler (1966) described these particles as being 120A wide, each probably made up of 4 subunits 60A deep, and a second class of single 60A particles. He suggested that these particles were composed of protein and lie outside but partially embedded in a double membrane of lipid. Bramberger and Park (1966), on the other hand, described these particles as being 170A and 90A in size and as being within the granal structure. To determine where the lipids were in this membrane, Bramberger and Park treated the chloroplast with an enzyme preparation from runner bean leaves which contained galactolipases and galactosidases. The chloroplasts were then observed under the electron microscope after freeze-etching. The results were compared with untreated chloroplasts. They suggested

that the thylakoid is built of a galactolipid layer on which large particles (175 A) in an embedding matrix are located. They also concluded that these 175 A particles contained lipid, protein and the chlorophyll.

It is fairly well established that all chloroplasts which perform the Hill reaction have the same lipid composition, even though the individual fatty acid composition shows slight variations. This similarity may indicate that these lipids are acting as specific structural components. Rosenberg (1967) postulated that the lipids represent specific structural components which serve to maintain the chlorophylls in correct steric orientation with one another and their associated enzymes in the chloroplast membrane. He suggested that this is made possible by a lock-and-key fit between the methyl groups in the phytol portion of the chlorophyll molecule and the methylene-interrupted, cis double bonds of the fatty acids in the galactosyl diglycerides. Induced polar interaction of double bonds with methyl groups was proposed as a force that favors binding of the two components. London-Van der Waals forces were also considered by Rosenberg to contribute to this binding.

Van Deenen and Haverkate (1966) proposed that the lipids such as the galactosyl diglycerides which are devoid of charged groups may participate in hydrophobic interactions with structural protein of the chloroplast while the negatively charged lipids such as the phosphatidyl glycerol and sulpholipid may play a prominent part in attaining charge-charge interactions between lipid micelles and proteins.

MATERIALS AND METHODS

Plant Material

Kinghorn wax beans (Phaseolus vulgaris) were chosen for the study because of their uniform and rapid rate of growth and the relative ease of chloroplast preparation. The beans were grown for 12 days in a growth chamber at 70°C with a light intensity of 1,900 foot candles and a photoperiod of 16 hours. Prior to harvest for chloroplast preparations the seedlings were placed in darkness for 12 hours to reduce the starch content in the chloroplasts. The leaves were harvested on the morning of the isolation of the chloroplasts.

Isolation of Chloroplasts

Approximately 100 gm of leaf material were ground with 150 ml of media containing 0.33 M sucrose and 0.1 M phosphate buffer at pH 6.8 in a mortar at 4°C. The pulp was squeezed in 4 layers of cheese cloth to separate the crude homogenate. The homogenate was centrifuged at 100 g for 2 minutes to remove the nuclei and cell debris. The supernatant was then centrifuged at 1,000 g for 15 minutes to yield the crude chloroplast pellet.

Purification of Chloroplasts

The crude preparation was washed by suspending the crude pellet in 0.33 M sucrose phosphate buffer and centrifugation at 3,000 g for 20 minutes. The resultant pellet of chloroplasts (plus some mitocondria) was then purified by resuspending the pellet in

0.33 M sucrose phosphate buffer and layering it on a discontinous sucrose density gradient. The gradient consisted of 3 sucrose phosphate buffers. The first layer was 1.0 M sucrose, the second 1.5 M and the third was 2.0 M sucrose, all in a 0.1 M phosphate buffer. Following centrifugation at 25,000 g for 20 minutes the chloroplast layer was retained at the interface between the 1.0 M and 1.5 M sucrose bands. The liquid above this layer was removed with a syringe and discarded. The chloroplast layer was then removed in a similar manner. An equal volume of sucrose-free phosphate buffer was added to the chloroplast layer and the suspension was centrifuged at 3,000 g for 15 minutes. The pellet obtained from this centrifugation was considered as the purified chloroplast pellet, (Fig. 1).

Chlorophyll Determination

The purified chloroplast pellet was brought to 100 ml with distilled water and mixed well to give a uniform suspension. An aliquot of the suspension (0.5 ml) was pipeted into a 25 ml volumetric flask to which was added 4.5 ml of water and 20 ml of acetone. The mixture was centrifuged at 3,000 g for 5 minutes. Absorption readings of the supernatant were made of a Beckman Model DK-1 recording spectrophotometer at 645 and 663 nm. Total chlorophyll as mg of chlorophyll per ml of chloroplast suspention was calculated according to Arnon (1949). The remainder of the chloroplast suspension was transferred to a 250 ml flask and freeze-dried over night.

Lipid Extraction

The lipids were extracted from the freeze-dried chloroplasts

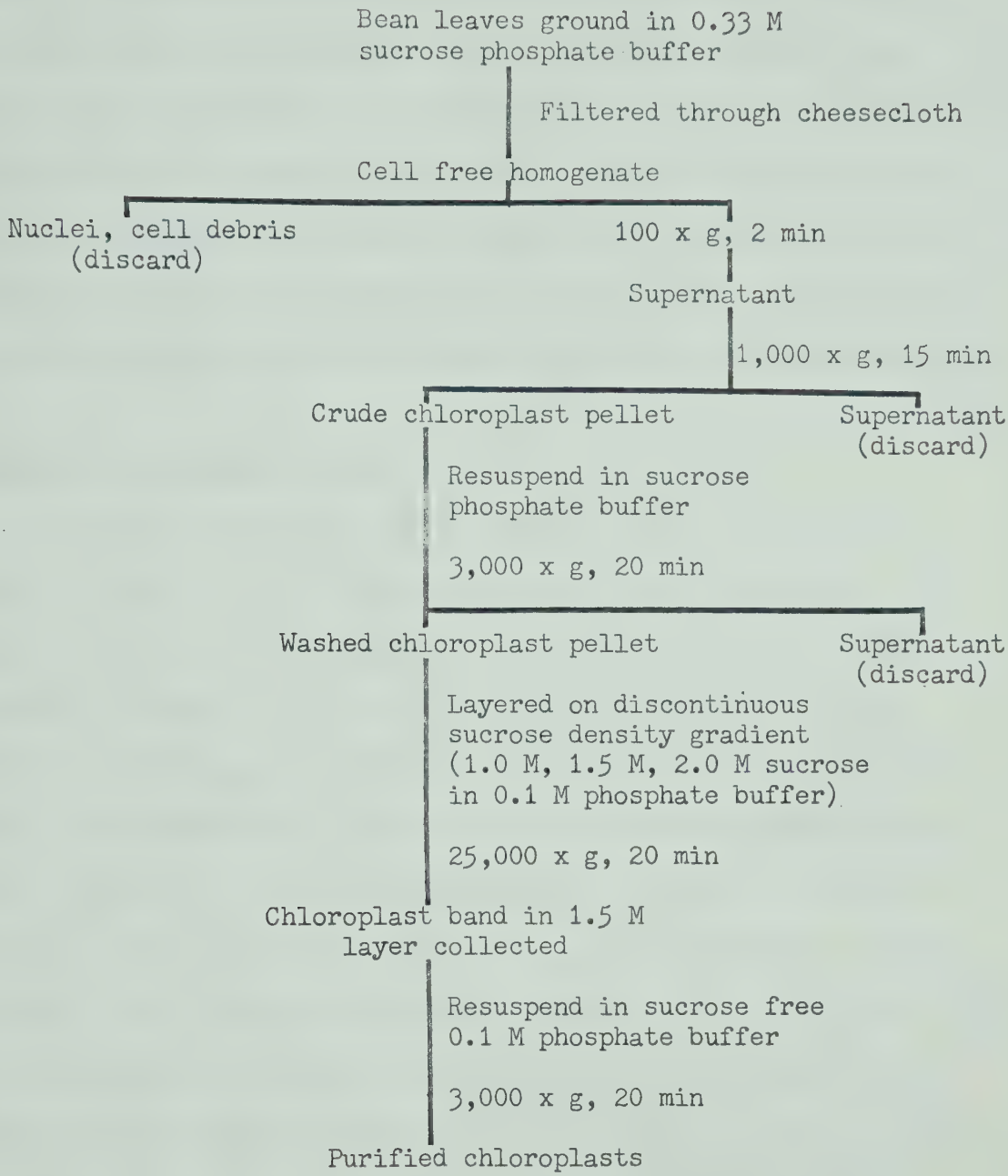

Fig. 1. Flow diagram of the isolation and purification of the chloroplasts from Kinghorn wax bean leaves. All operations were at $4^{o}C$ in 0.1 M phosphate buffers (pH 6.8), containing 0.01 M KCl and 0.01 M $MgCl_2$.

with 2:1 (v/v) chloroform-methanol and the extract was filtered through a fritted glass Buchner funnel and evaporated to a small volume on a flash evaporator. The extract was then filtered through anhydrous sodium sulphate to remove any traces of water and was evaporated to dryness on a flash evaporator at room temperature. The residue was flushed thoroughly with nitrogen, redissolved with 3:1 chloroform-methanol (C/M) and brought to exactly 5 ml in volume.

Purification of Lipid Extracts

Purification of the lipid extract was carried out on a Sephadex column by the method of Maxwell and Williams (1967). The fines were removed from Sephadex LH-20 by suspending it in water, allowing to settle for 15 minutes and then decanting the liquid. This was repeated several times to remove chloroform-insoluble residue. The Sephadex was then filtered in a Buchner funnel and air-dried. Before use, the gel was soaked in 3:1 C/M for a minimum of 48 hours. The slurry was poured into a 1 by 18 cm column. Because Sephadex LH-20 floats in this solvent mixture, a flow rate had to be maintained while the column was being poured. After the column was prepared without packing, a disc of filter paper was placed on top of the gel to reduce the tendency of the gel to float when further solvent was added.

A 1 ml sample of lipid extract in 3:1 C/M was pipeted carefully onto the filter paper disc and washed into the column with very small portions of solvent. To ensure good separation of lipids from contaminats a flow rate of approximately 0.1 ml per minute was used. A water jacket around the column and a constant temperature

water bath were employed to keep the column at a constant temperature of 20° C.

One ml fractions were collected and assayed for total sugars by the method of Dubois et al. (1956). The fractions were dried under nitrogen and were hydrolyzed at 100° C with 2 ml of 3 N sulfuric acid for 2 hours. After cooling, 1 ml of 5 per cent phenol and 5 ml of conc. sulfuric acid were added to each test tube. The tubes were mixed on a vortex mixer and then allowed to stand at room temperature for 15 minutes before measuring the absorbance on a Beckman DK-1 spectrophotometer at 490 nm. (this is the region of maximum absorbance of the solution, as shown in Fig. 2). The concentrations of sugars were calculated from a standard curve for galactose (Fig. 3).

In order to determine the amounts of water soluble sugars, 1 ml fractions were collected from an identical column. They were dried on a flash evaporator, redissolved in 1 ml with chloroform and then washed thoroughly with 2 ml of water by mixing the contents of the centrifuge tube on a vortex mixer. The two layers were separated by centrifugation at 1,000 g for 3 minutes and 0.5 ml aliquots of the water layer were removed for analysis of water-soluble components. Water-soluble sugars were determined as galactose by the method of Dubois et al. (1956). The difference between the values obtained for the total sugar and the water-soluble sugar was taken as representing the amount of lipid sugar and is reported as galactose. The fractions of the column that were found to contain the lipids on the basis of lipid sugar content were then combined into a single fraction. This fraction, which also contained pigments, was dried on a flash

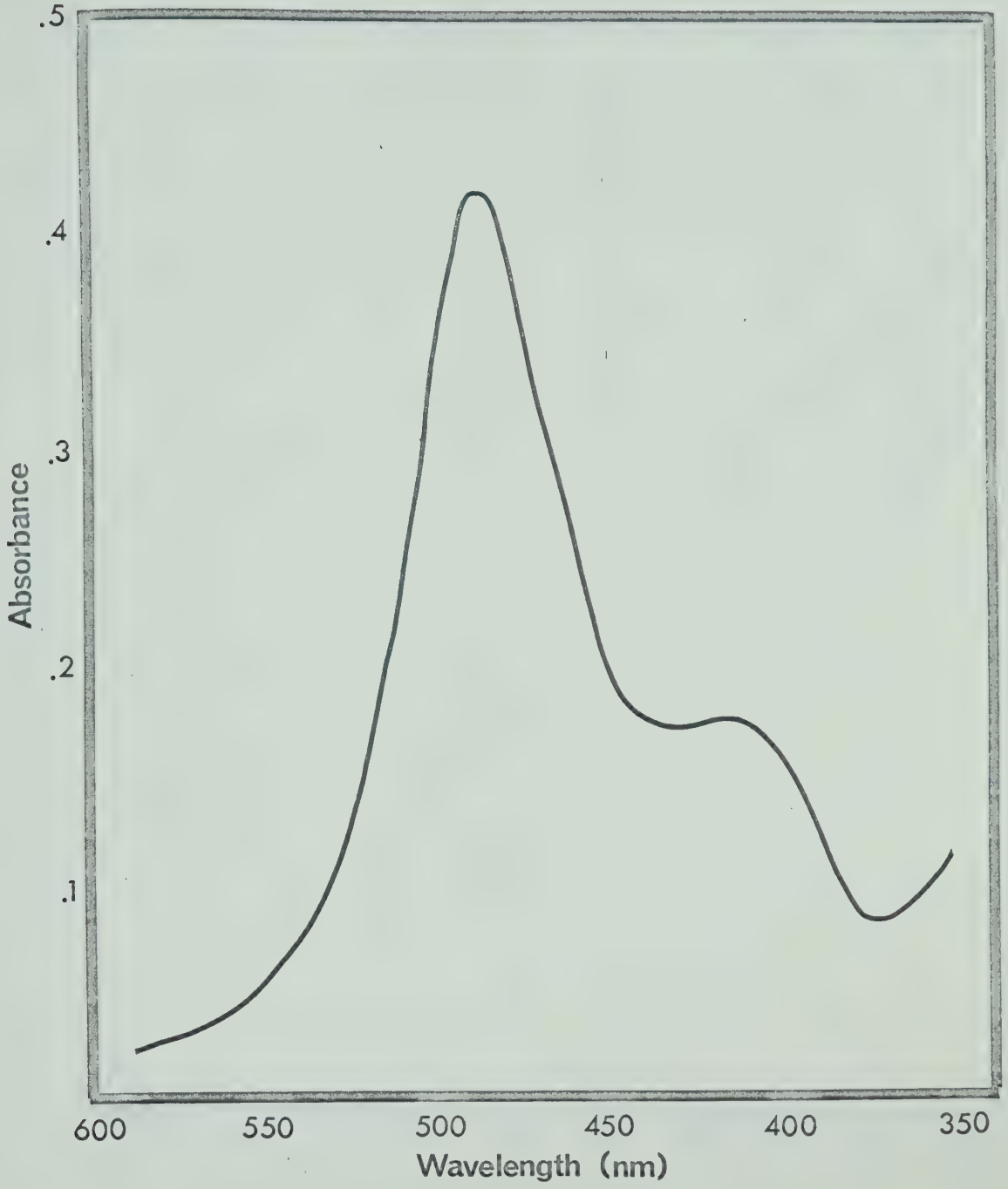

Fig. 2. Absorption Spectrum of Galactose in Phenol/Sulfuric Acid.

To 1 ml containing 100 ug galactose were added 1 ml 5% phenol and 5 ml concentrated sulfuric acid. After mixing and cooling to room temperature, the absorption spectrum was read on a Beckman DK-1 spectrophotometer.

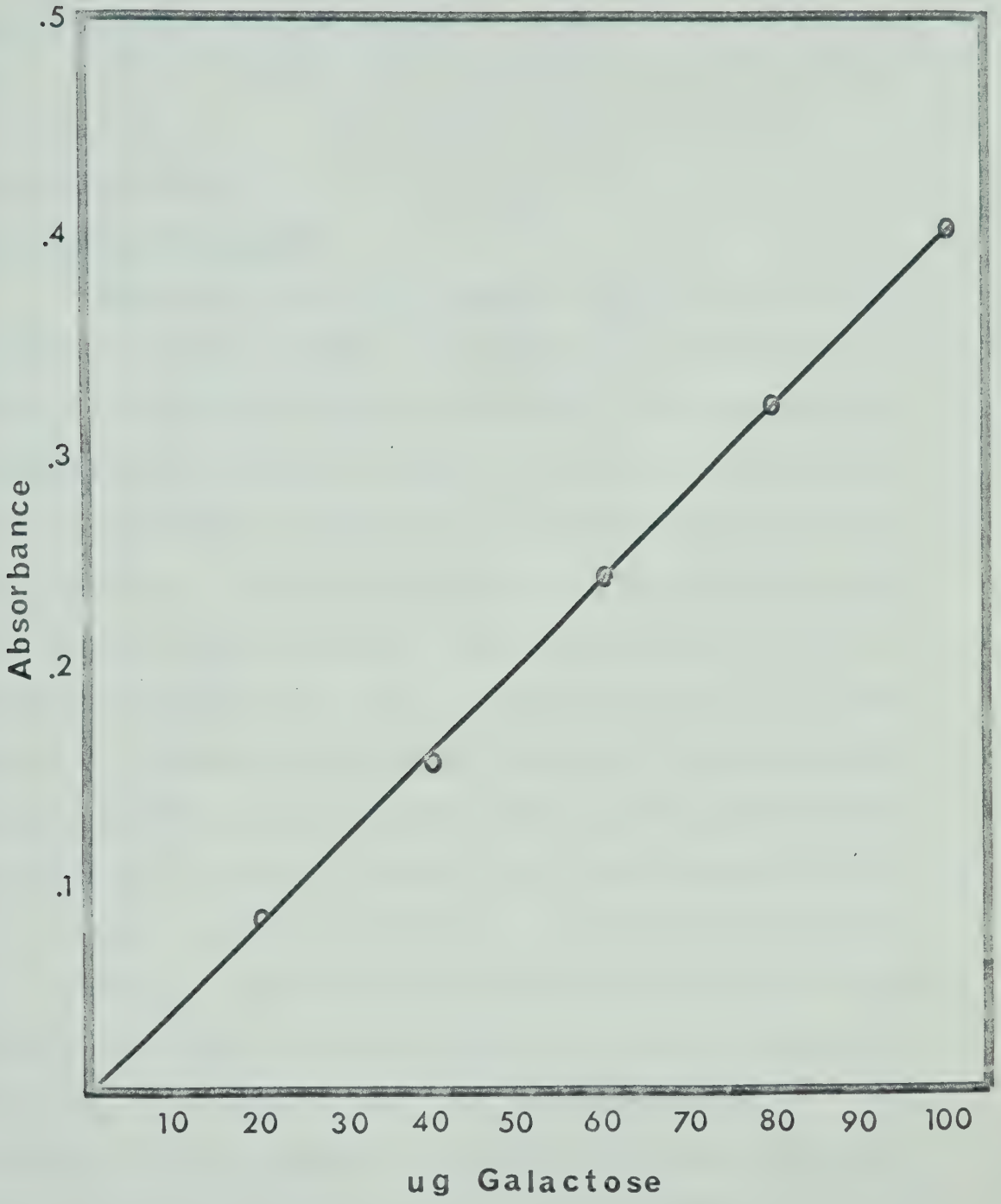

Fig. 3. Galactose Standard Curve Used to Estimate the Concentrations of Sugar in Eluates from Sephadex LH-20 Columns. The readings were taken at 490 nm.

evaporator, redissolved and brought to a volume of 5 ml with chloroform.

Separation of Lipids

1. Column Chromatography

Silicic acid columns were prepared by the method described by Maxwell and Williams (1968). The silicic acid (100 mesh) was washed five times in distilled water to remove fines and was then thoroughly dried in an oven at 110° C. The silicic acid was suspended in chloroform and poured into a column 1 cm by 1.75 cm high without packing. A filter paper disc was placed on top of the gel to prevent any disturbance of the column during solvent additions. The column was then washed with 5 to 10 ml of chloroform. A 1 ml aliquot of lipid in chloroform was applied to the column and washed carefully with small quantities of solvent. A water jacket around the column and a constant temperature water bath were used to keep the column at a temperature of 20° C. It was found that the column had to be kept at a constant temperature to get reproducible results. Fraction 1 was eluted with 25 ml chloroform. Fraction 2 was eluted with 25 ml 0.4 per cent acetic acid in chloroform. The flow rate of fractions 1 and 2 was approximately 0.3 ml per minute. Fraction 3 was eluted with 40 ml 0.4 per cent acetic acid and 3 per cent methanol in chloroform. Fraction 4 was eluted with 25 ml 0.4 per cent acetic acid in 1:1 C/M. A faster flow rate was used in the elution of fractions 3 and 4. Fractions 3 and 4 were evaporated on a flash evaporator, flushed with nitrogen and redissolved in chloroform.

2. Thin-Layer Chromatography

Thin layer plates coated with approximately 250 μ of silica gel HR (Merck) were activated at 120° C for 30 minutes immediately before use and developed with the solvent system Chloroform/methanol/ glacial acetic acid/ water (85/15/10/3) in tanks lined with filter paper. Initially fractions 1, 2, 3 and 4 were each spotted on a plate and the plate developed in the solvent system for 50 to 60 minutes. After drying the plates for 5 to 10 minutes they were sprayed with a solution of Rhodamine 6G prepared on the day of use by mixing 1 ml of a stock solution of 0.1 per cent (w/v) Rhodamine 6G in methanol with 25 ml of 2 N ammonium hydroxide. The sprayed plates were viewed while still damp under long-wave UV light. The lipids were distinguishable by their pink fluorescence. The identification of sugar-containing spots was made by spraying the plates with 0.2 per cent anthrone (w/v) in concentrated sulfuric acid followed by heating at 70° C (Rosenberg, Gouzux and Milch, 1966). This produced green spots with galactosyl diglycerides and violet spots with sulpholipids.

Subsequently, fractions 3 and 4 were applied as 4 cm bands on the plates. Development of the chromatogram was complete in 50 to 60 minutes and after drying for 5 to 10 minutes the plates were subjected to iodine vapor which made the bands visible. The bands were outlined with a needle and the iodine was allowed to evaporate. The bands were then scraped off the plates into glass centrifuge tubes with a razor blade.

Estimation of Sulpholipid

In addition to scraping off the band from the thin-layer plate which corresponded to sulpholipid on the basis of Rf, a blank was also taken from a comparable zone on the plate. To each centrifuge tube (sample and blank) 2 ml of 2 N sulfuric acid was added. The tubes were then loosely stoppered with glass marbles and placed in boiling water bath for 60 minutes. During this time the tubes were shaken four or five times. After cooling and centrifugation at 2,000 g for 5 minutes, a 1 ml aliquot was withdrawn from each tube and transferred to test tubes. After trying varying amounts of phenol and sulfuric acid, it was found that one ml of 2 per cent phenol and 4 ml of conc. sulfuric acid gave the maximum absorbance. Thus, these were the amounts added to each aliquot. The tubes were mixed on a vortex mixer and then were allowed to stand at room temperature for 15 minutes before measuring the absorbance of the sample at 490 nm. The readings were compared to a standard galactose curve (Fig. 4). Galactose was substituted for sulphoquinovose as a standard in this assay. Equal weights of galactose and sulphoquinovose give the same net absorbance when using the above method (Roughan and Batt, 1968). To convert the galactose figures to sulpholipid, the experimentally determined values were multiplied by the factor 4.5. This value is derived from the molecular weight of sulphoquinovosyl diglyceride and is based on the assumption that the sulpholipid contained equal amounts of palmitic acid and linolenic acid.

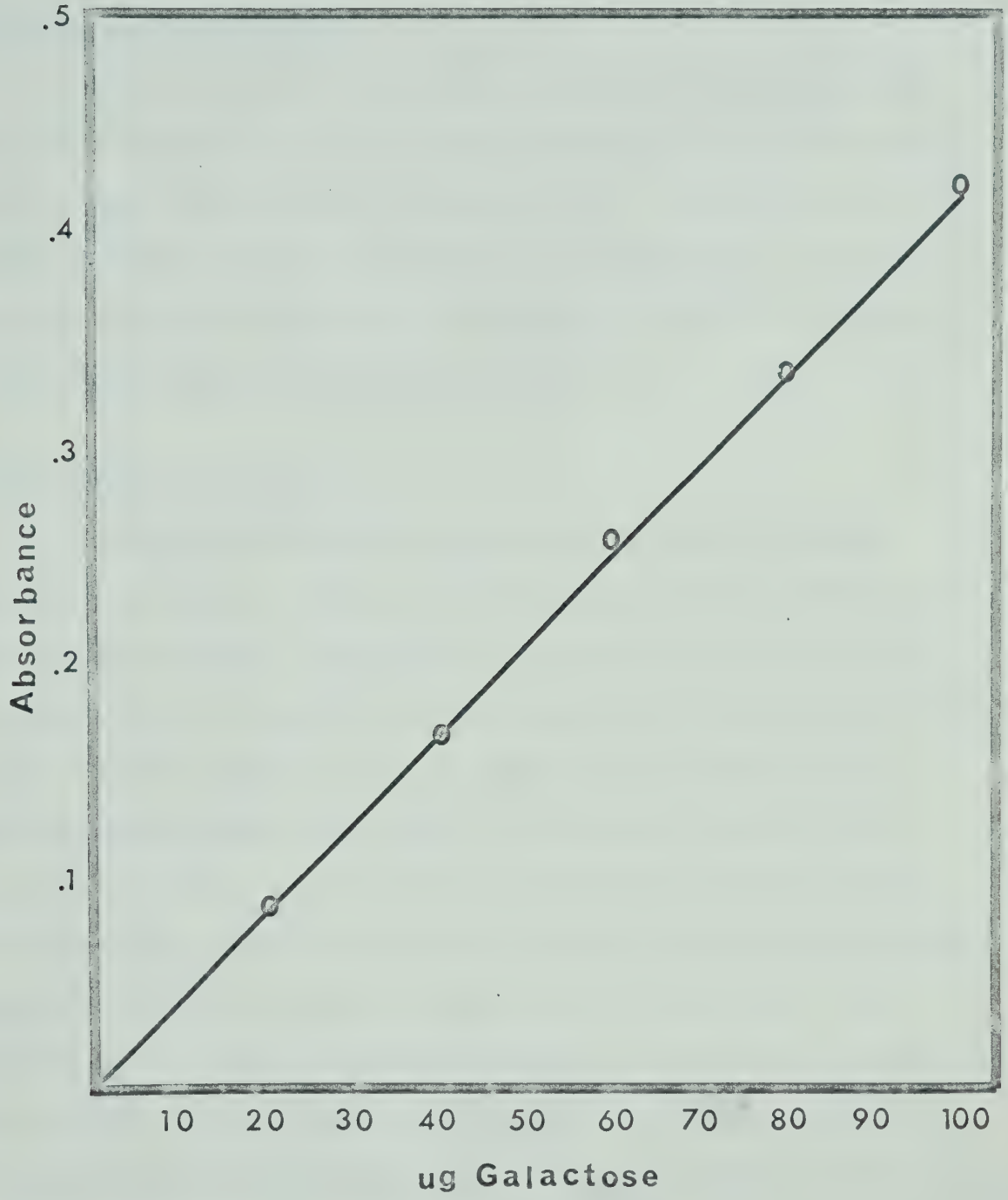

Fig. 4. Galactose Standard Curve Used to Estimate Sugar in Lipids Separated on Silica Gel HR Plates. The solutions contained a range of concentrations of galactose in 0.5 ml, 1 ml 2% phenol and 4 ml concentrated sulfuric acid. The readings were taken at 490 nm.

Estimation of Galactolipids

The monogalactosyl diglyceride and digalactosyl diglyceride bands were treated like that of sulpholipid but 0.5 ml of the sample was used after centrifugation instead of 1 ml. To convert these galactose figures to mono- and digalactosyl diglyceride, the experimentally determined values were multiplied by factors of 4.3 and 2.7 respectively (Maxwell and Williams, 1968).

Phospholipid Determinations

Phosphorus determinations were carried out by the method described by Bartlett (1958). A 0.5 ml aliquot of the purified lipid was used for analysis. The aliquot to be analyzed and 0.5 ml of 10 N sulfuric acid were placed in a 20 ml pyrex test tube and heated in a 150 to 160° C oven for 3 hours. Several drops of 30 per cent hydrogen peroxide were added and the solution was returned to the oven for 1.5 hours more to complete the combustion and to decompose all the peroxide. To the hydrolysate 4.6 ml of 0.22 per cent ammonium molybdate and 0.2 ml of Fiske-Subba Row reagent were added. The contents of the tubes were mixed thoroughly, the tubes were covered with glass marbles and heated for 7 minutes in a boiling water bath. The optical density at 830 nm (which is the region of maximum absorbance of the phosphomolybdate complex, as shown in Fig. 5) was recorded with a Beckman DK-1 spectrophotometer. The concentration of phosphorus in the sample was then determined from a standard curve (Fig. 6). To convert the phosphorus figures to phospholipids the values were multiplied by the factor 24. This value is based on the

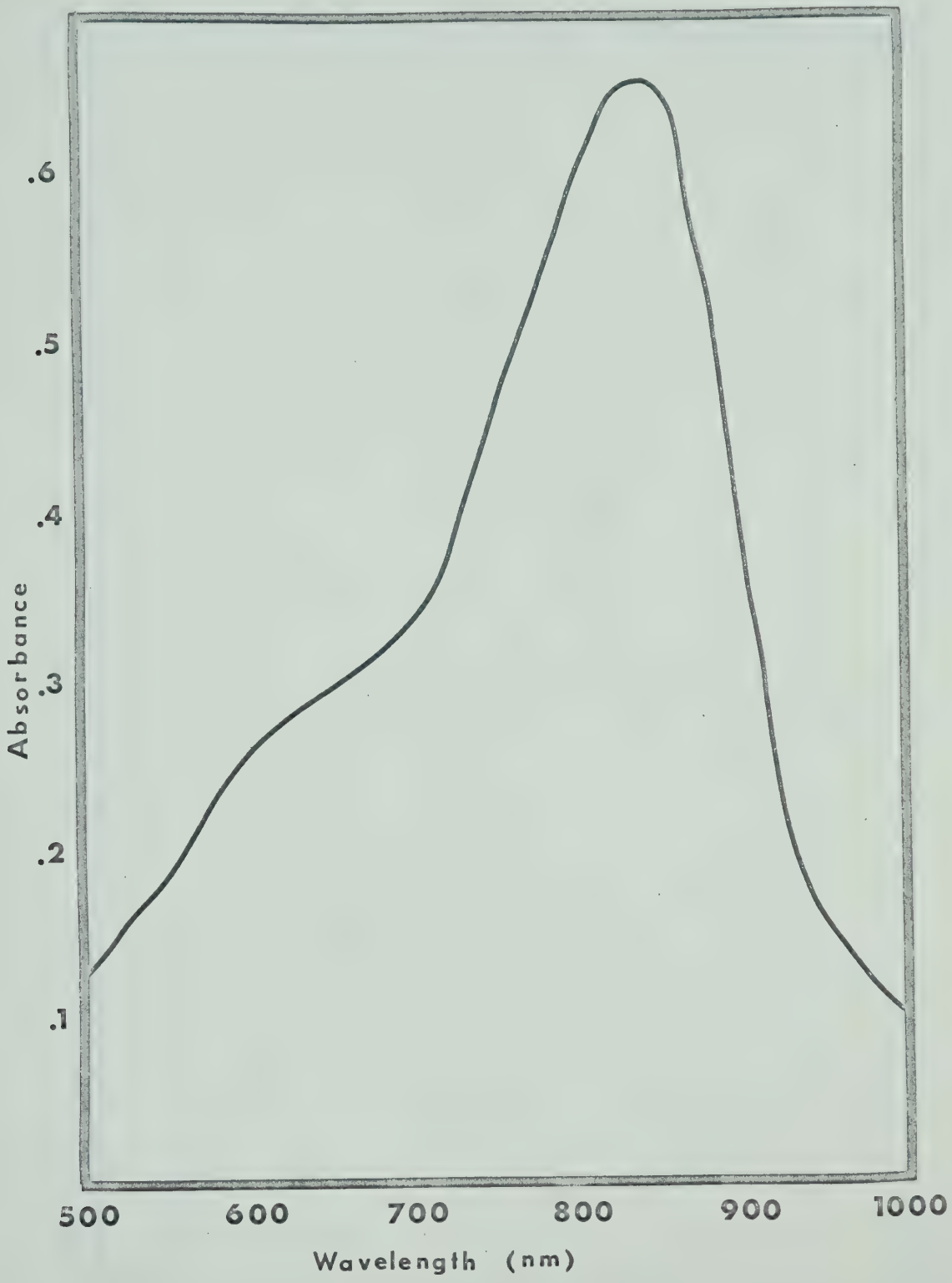

Fig. 5. Absorption Spectrum of Phosphomolybdate Complex as Recorded With a Beckman DK-1 Spectrophotometer.

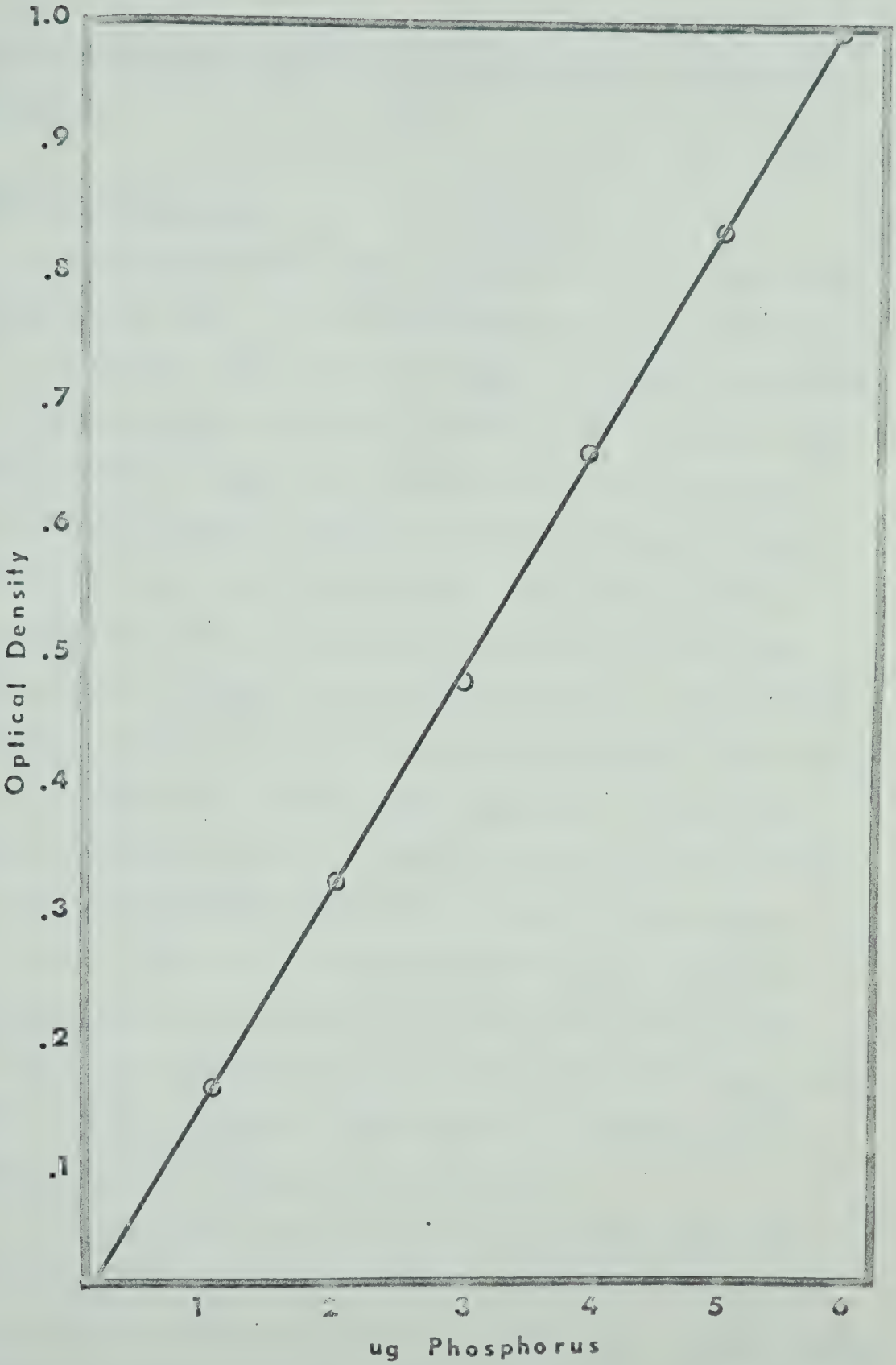

Fig. 6. Standard Curve for Phosphorus. The readings were taken at 830 nm.

assumption that the phospholipids are predominantly phosphatidyl glycerol.

Fatty Acid Composition

The glycolipid bands were scraped off the thin-layer plates into glass test tubes. To each tube were added 2 ml of methanol and 0.1 ml of sulfuric acid. After refluxing for one hour, the contents of the tube were diluted with 3 ml of water. The esters were then extracted with petroleum ether. The methylated fatty acids were dried by evaporating the petroleum ether under nitrogen and the methyl esters were taken up in methanol. The fatty acid methyl esters were analyzed on an Aerograph model 200 gas chromatograph equipped with a hydrogen flame ionization detector. A coiled stainless steel column 10 feet by 1/8 inches was packed with diethylene glycol succinate (20%) on 60-80 mesh chromosorb P. The ends were plugged with glass wool and the column was connected to the injector end of the Aerograph 200. Nitrogen was allowed to pass through the column at a flow rate of 25 ml per minute and the column was conditioned for 48 hours at 200° C. It was then connected to the detector end and used for separation of the esters. The column temperature was 195° C, injector temperature 240° C, and the flow rate of nitrogen was maintained at 25 ml per minute.

The peaks were identified by matching unknown fatty acids with known standards. The standard fatty acids were obtained from Applied Science Laboratories, inc. State College, Pa. The areas under the peaks were measured with a planimeter.

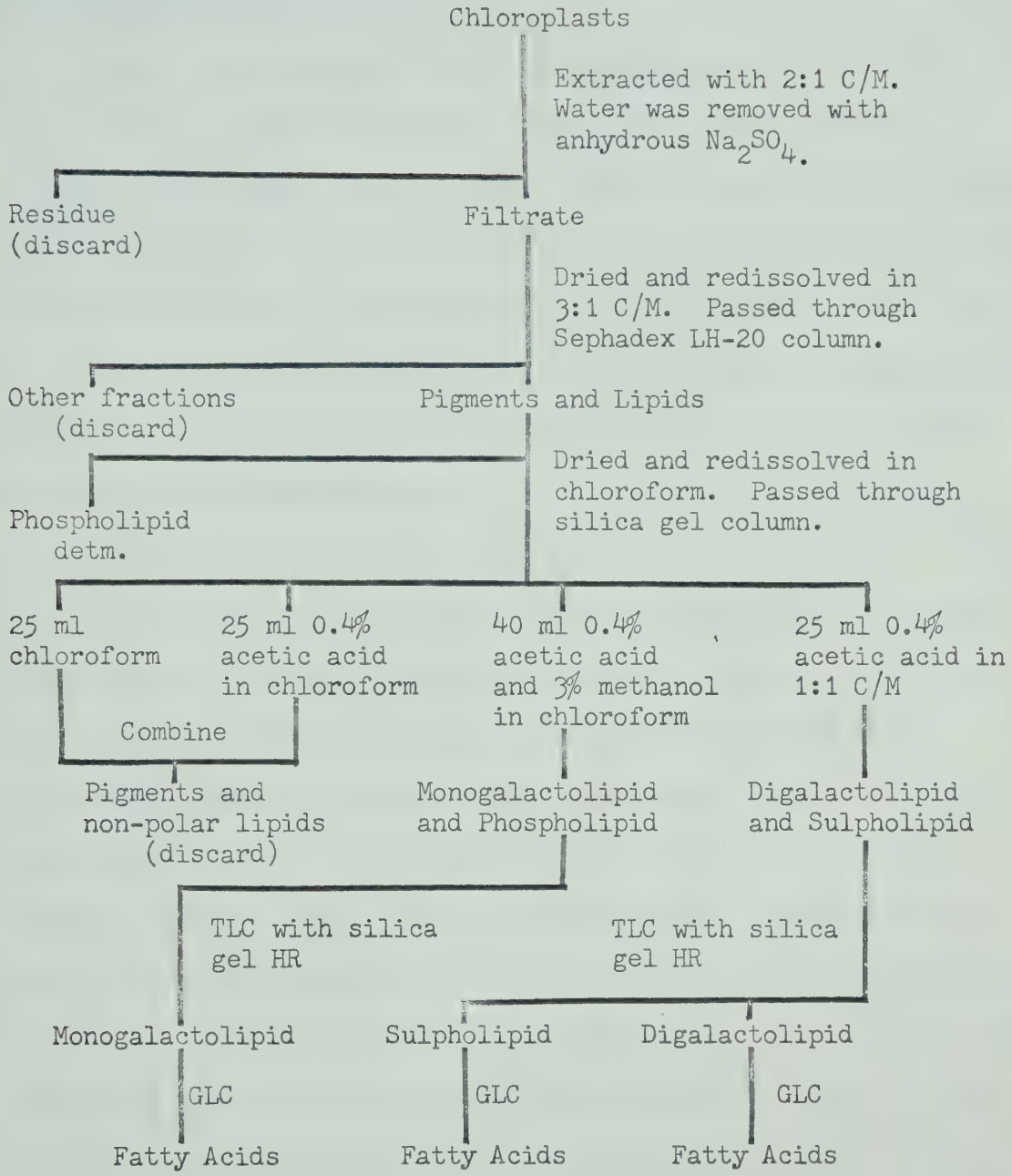

Fig. 7. Flow diagram of the extraction, purification and analysis of the lipid components of Kinghorn wax bean chloroplasts.

RESULTS AND DISCUSSION

Lipid Extraction and Purification

There are various methods for extracting total lipids from plant tissue. Solvents which have been used include n-butanol, petroleum ether, ethyl ether, acetone, chloroform and ethanol. Water saturated n-butanol is a very satisfactory solvent for extraction but its high boiling point may cause oxidation of unsaturated fatty acids when further analysis is necessarary. A 2:1 chloroform-methanol solvent mixture originally proposed by Folch, Lees and Stanley (1957) was used in this investigation.

When solvents containing methanol are used during lipid extraction of plant tissue, the artifact, phosphatidyl methanol is formed by the action of phospholipase D on endogenous phospholipids (Yang, Freer and Benson, 1967). A problem associated with this is that phosphatidyl methanol has been shown to have very similar thin-layer chromatographic properties to those of diphosphatidyl glycerol (Renkonen, 1968). Recent studies have shown that only phosphatidyl choline and ethanolamine are transesterified to phosphatidyl methanol during room temperature lipid extraction of white clover, pumpkin and cauliflower leaves with methanol (Roughan and Batt, 1969). In the present study, phospholipids were determined only as total phospholipids and assumed to be entirely phosphatidyl glycerol. Therefore the transesterification of these phospholipids in this case is no problem.

Most of the earlier work in the analysis of plant lipids involved separation methods such as paper chromatography (Wintermans,

1960), silica gel impregnated paper (Kates, 1960) and thin layer chromatography (Nichols, 1963; Lepage, 1964). However, these methods were limited in efficiency of the separations obtained. The more recent methods include absorption chromatography on silicic acid, florisil, activated carbon, DEAE cellulose and Sephadex columns, alone or in combination with thin layer chromatography. Silicic acid columns are most widely used even though the early attempts in their use were not successful because of the contamination problem with chlorophyll and poor separations. The methods used in this study involve the combination of Sephadex LH-20 column with silicic acid column chromatography and thin-layer chromatography.

Since this method of determining the glycolipids analytically is by a sugar estimation (Dubois, Gilles, Hamilton, Rebers and Smith, 1956), sucrose from the chloroplast isolation media is a significant contaminant in the lipid extracts of the chloroplasts. An efficient way of separating the lipid sugars from the water-soluble or free sugars is to use a Sephadex column and Sephadex LH-20 can be used when applying organic solvents. The sugars, having a lower molecular weight than the lipids, move through the column at a slower rate. The results of assays of both water-soluble and lipid sugars eluted from the Sephadex LH-20 column used are presented in Table I. In Fig. 8 the elution patterns are presented graphically. Fractions 1 to 4 were found to contain a high amount of free sugar relative to the subsequent samples. Since it is not possible for sample components to be eluted until a volume of liquid greater than the void volume of the gel has passed through the column, it is suspected this was due

Table I. Distribution of Lipid and Non-lipid Sugars Isolated from Bean Chloroplasts.

Fraction No.	Sugars as ug galactose / ml		
	Total Sugars	Water-soluble Sugars	Lipid Sugars*
1	10.3	10.3	0
2	5.0	5.0	0
3	4.0	4.0	0
4	4.0	4.0	0
5	3.5	3.5	0
6	7.5	3.0	4.5
7	122.4	3.6	118.8
8	770.0	9.2	760.8
9	840.0	5.4	834.6
10	62.6	5.0	57.6
11	23.0	3.1	19.9
12	9.5	8.3	1.2
13	3.0	2.1	0.9
14	4.0	3.2	0.8
15	3.6	3.5	0.1
16	12.4	12.0	0.4

One ml aliquots containing 2,625 ug total sugar (as galactose) were applied to Sephadex LH-20 columns and eluted with chloroform-methanol (3:1) at a flow rate of 0.1 ml per min. The temperature of the column was maintained at 20°C.

* Calculated by subtracting water-soluble sugars from total sugars.

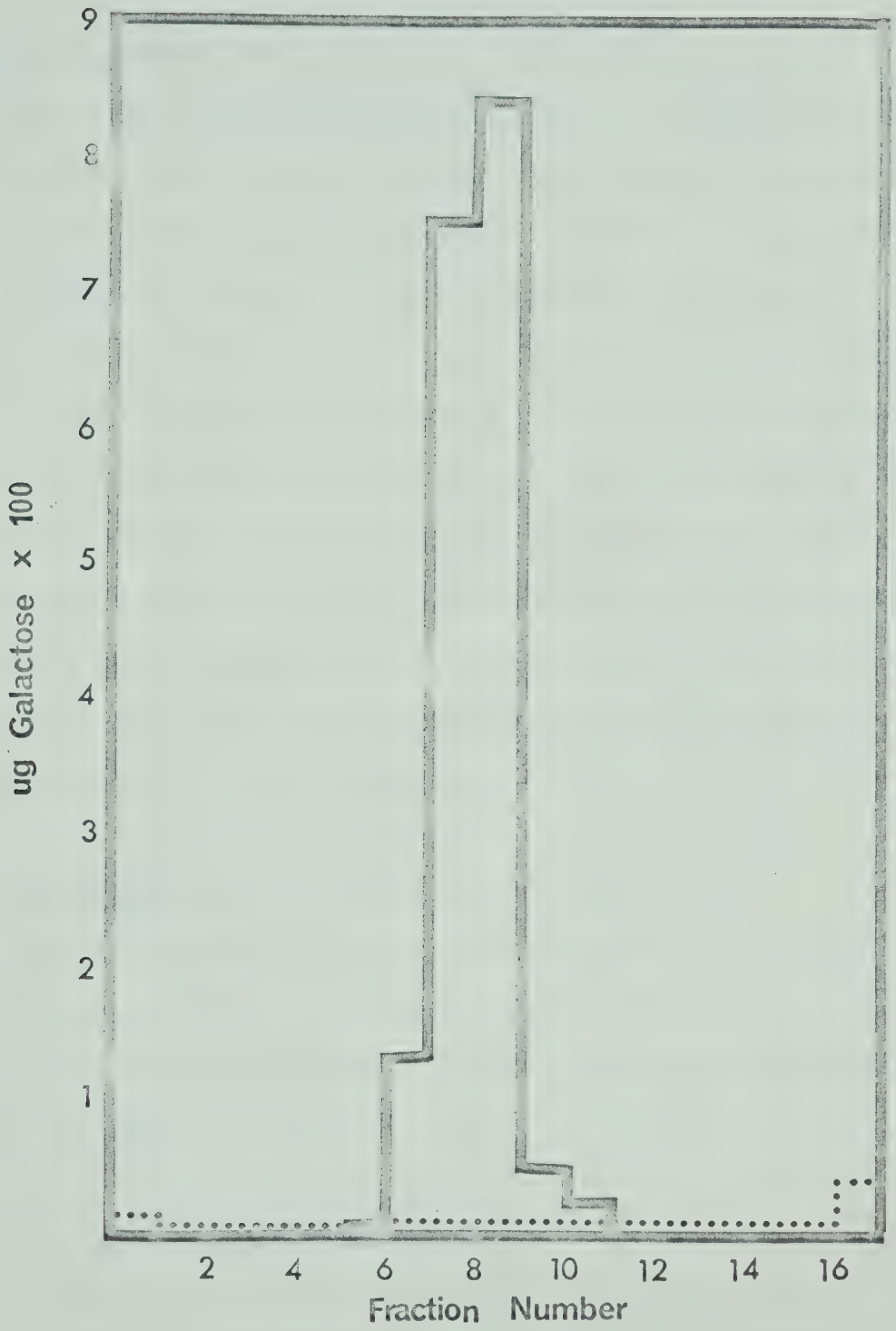

Fig. 8. Distribution of lipid sugars and non-lipid sugars expressed as ug galactose/fraction, from a Sephadex LH-20 column.

Lipid sugars

Water-soluble sugars

to residual Sephadex fines.

The sugar-containing lipids were found to be eluted in fractions 6 to 11. This combined volume of lipid fractions was the same as the fractions which contained pigments. Therefore the pigments were used later as a marker to indicate where the lipid was on the column. Soluble sugars started to elute in fraction 16 and eluted in a much wider band which lagged behind the lipid band by several millilitres, thus ensuring good separation. Glycolipid recovery in fractions 6 to 11 was 90 per cent. A disadvantage of this method is that some of the sugars are not eluted with the chloroform-methanol mixture used and a methanol-water mixture had to be used to clean the column. This causes the column to shrink and thus the column must be repoured before it can be used again.

Separation of Lipids

In order to assay the glycolipids on the basis of their sugar moieties, they must first be separated. This was done using silica gel column chromatography followed by thin-layer chromatography on silica gel HR.

A. Silicic Acid Column Chromatography

A short silicic acid column of only 1.75 cm was used to eliminate trailing which can cause poor separation on longer columns. Thin-layer chromatographic analysis on silica gel G of the silicic acid column eluates was used to indicate whether there was any contamination of one fraction with another (Fig. 9). When sprayed with anthrone-sulfuric acid, there was no indication of the presence of

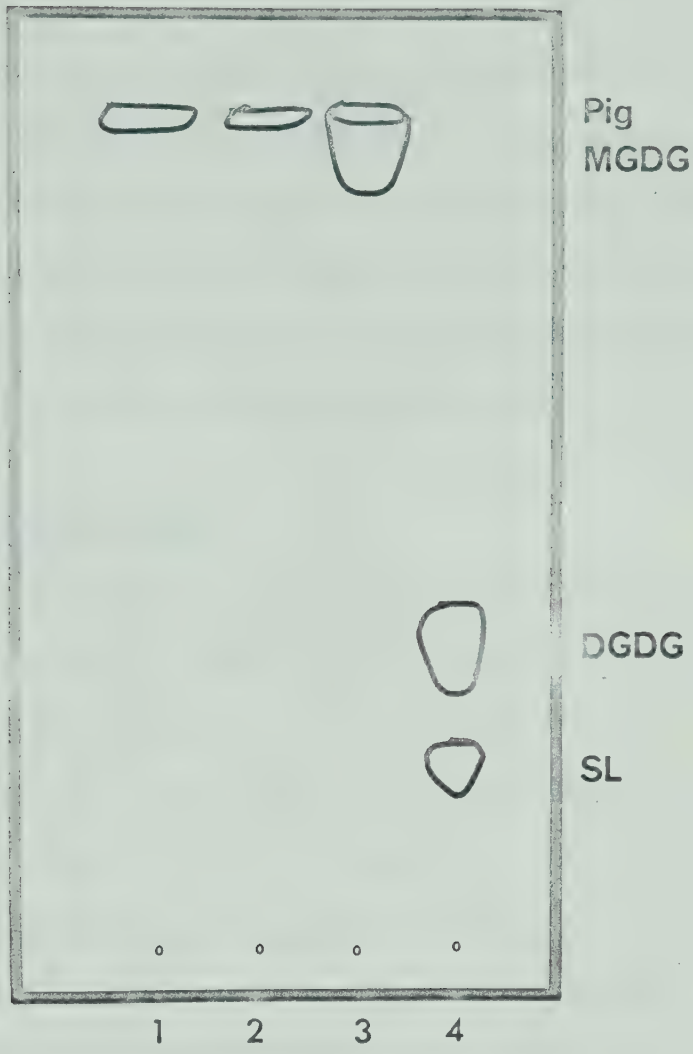

Fig. 9. Diagram of Silica Gel HR Plate Used to Separate the Components Eluted as Fractions 1, 2, 3 and 4 from the Silicic Acid Column. The developing solvent was chloroform/methanol/glacial acetic acid/ water (85/15/10/3). The plates were sprayed with 0.2% anthrone (w/v) in concentrated sulfuric acid and heated at 70°C. The diglycerides were identified on the basis of color and by comparing the Rf values with those of Roughan and Batt (1968). Pig, pigments; MGDG, monogalactosyl diglyceride; DGDG, digalactosyl diglyceride; SL, sulphoquinovosyl diglyceride.

glycolipids in fractions 1 and 2. Fraction 3 contained a glycolipid which was identified by its Rf value as being monogalactosyl diglyceride. Fraction 4 contained two spots which were identified by their Rf values as being digalactosyl diglyceride and sulphoquinovosyl diglyceride. Thin-layer chromatography thus confirmed that fractions 3 and 4 were distinctly different.

B. Thin-layer Chromatography

Since the silicic acid column was unsatisfactory for the separation of digalactosyl diglyceride from sulphoquinovosyl diglyceride, thin-layer chromatography on silica gel was required. Both silica gel G and HR were tried and both gave good separation but silica gel G was unsuitable for the analysis of the sulpholipid later. Fraction 3 from the silicic acid column was applied to a plate to separate out some of the chlorophyll that still remained. On a normal chromatogram the pigments appeared right below the solvent front and the monogalactosyl diglyceride immediately behind. There was still partial mixing of the pigments and the monogalactosyl diglyceride but accurate analyses were possible after acid hydrolysis of the lipid-loaded adsorbent, leaving the pigment and fatty acids adsorbed on the gel.

The digalactosyl diglyceride and sulphoquinovosyl diglyceride were also separated from fraction 4 on silica gel HR using the same developing solvent system.

Glycolipid and Phospholipid Analysis

The lipids were analyzed on the basis of either phosphate or

sugar obtained after hydrolysis of the isolated lipid.

The phenol-sulfuric acid estimation was found by Roughan and Batt (1968) to be one of the most sensitive methods for sugar analysis. When they used galactose and sulphoquinovose they found that this method was equivalent to that of the reducing sugar procedure of Dygert et al., and almost twice as sensitive as the anthrone estimation. They also found that the presence of silica gel did not affect the phenol-sulfuric acid reaction and did interfere with the reducing sugar procedure. On the basis of sensitivity and the fact that the lipids do not have to be eluted from the gel, the phenol-sulfuric acid method was used.

After galactose and the chloroplast glycolipids had been hydrolyzed in the presence of silica gel HR with 2 N sulfuric acid for 60 minutes, the characteristic absorbance vs. wavelength curves in Figure 10 were obtained on a Beckman DK-1 spectophotometer. One hour appeared to be long enough to liberate the sugar from the lipids, leaving the pigments and the fatty acids absorbed on the gel. Centrifugation at 2,000 g for 5 minutes was sufficient to clear the supernatant. An aliquot of the hydrolyzed lipids and galactose were treated separately by adding varying amounts of phenol and concentrated sulfuric acid to find the maximum absorbance. It was found, in all cases, that as the amount of phenol was decreased, the absorbance increased to a maximum where it leveled off. The maximum absorbance was observed when 1 ml of 2 per cent phenol and 4 ml of concentrated sulfuric acid were used. The maximum absorbance in all cases was at 490 nm. It was also found that when the glycolipids were separated

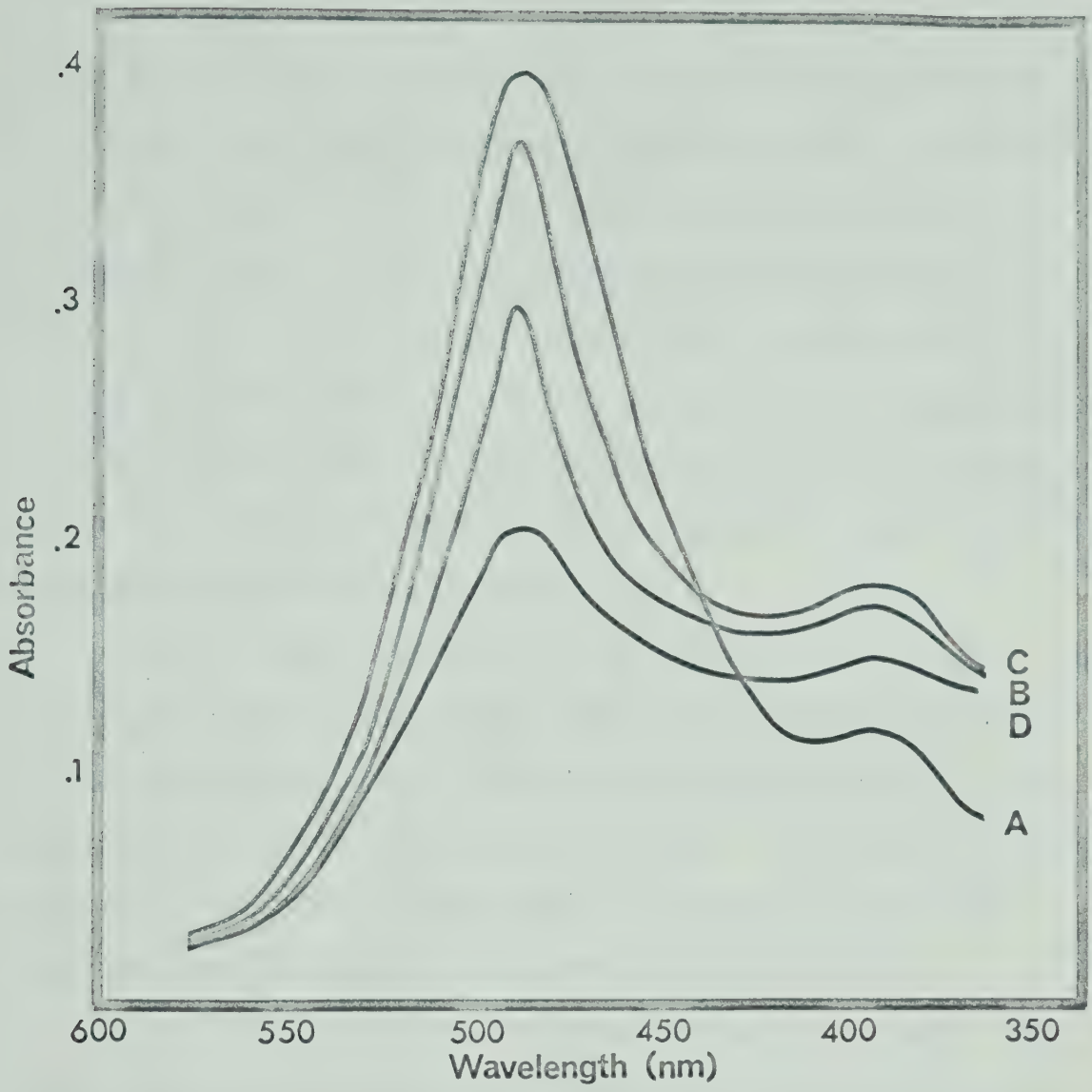

Fig. 10. Absorption Spectra of Glycolipids Isolated from Kinghorn Wax Bean Chloroplasts and of Galactose. (A) galactose; (B) monogalactosyl diglyceride; (C) digalactosyl diglyceride; (D) sulphoquinovosyl diglyceride. The galactose standard was prepared by adding 1 ml of the standard solution (100 ug/ml) to a centrifuge tube containing a small amount of silica gel HR. To this 1 ml of 4 N sulfuric acid was added and the solution was heated at 100°C for 60 minutes. After cooling and centrifuging down the silica gel, a 1 ml aliquot was withdrawn from the tube and 1 ml 2% phenol and 4 ml conc. sulfuric acid were added. The solution was shaken and cooled to room temperature before scanning on a Beckman DK-1 spectrophotometer. The glycolipids were scraped off the silica gel HR plates into centrifuge tubes and 2 ml of 2 N sulfuric acid was added. They were then treated the same as the standard.

on silica gel G, that the blanks were variable and gave poor results. On the other hand, silica gel HR gave consistant results even when varying amounts were used. It was then established that when 1 ml of 2 per cent phenol and 4 ml of concentrated sulfuric acid were used in the presence of silica gel HR, a linear standard curve could be produced between 10 to 100 ug of galactose at a wavelength of 490 nm. Such a standard curve was used (Fig. 4) for the estimation of sugar concentrations from the bean chloroplast glycolipids which were separated on silica gel HR plates.

Table II shows the results of the analysis in terms of chlorophyll content of the tissue. The results show that the chloroplast is particularly rich in the galactosyl diglycerides with monogalactosyl diglyceride being the most abundant. The only other major acyl lipids found were a sulpholipid and a phospholipid which has been identified as phosphatidyl glycerol on the basis of Rf.

Some reports of the distribution of lipids in photosynthetic tissue have been made previously. The ratios of the glycerolipids from this study and previous studies by other workers are compared in Table III. It may be seen that in all cases the galactosyl diglycerides are the most abundant and the sulpholipid is present in least amounts. The galactolipid results for the wax bean chloroplasts of this study appear higher compared to the other studies. This may be a consequence of lower concentrations of sulpholipid in the wax bean chloroplasts. The time of harvest may have some effect on the concentration of sulpholipid. Roughan and Batt (1969) have suggested that the sulpholipid levels of red clover leaves may be reduced three-

Table II. Acyl Lipids of Kinghorn Wax Bean Chloroplasts.

Lipid	ug/mg chlorophyll	uM/mg chlorophyll
Monogalactosyl diglyceride	569.6 ± 18.3	0.74 ± 0.03
Digalactosyl diglyceride	491.5 ± 57.7	0.53 ± 0.06
Phospholipids	128.0 ± 9.2	0.17 ± 0.01
Sulphoquinovosyl diglyceride	37.7 ± 5.1	0.05 ± 0.005

The results are the means ± half range of 3 replications. The glycolipid levels were calculated from the sugar moities which were estimated quantitatively spectrophotometrically. The multiplication factors used were 4.3 for monogalactosyl diglyceride, 2.7 for digalactosyl diglyceride, and 4.5 for sulphoquinovosyl diglyceride. The phospholipid value was derived by quantitatively assaying the amount of phosphorus spectrophotometrically and multiplying the value by 24.

Table III. Comparison of Acyl Molar Ratios of Bean Chloroplasts With Other Plant Tissues.

	Wax Bean Chloroplast	Spinach (1) Chloroplast	Spinach (2) Lamellae	Photosynthetic(3) Tissues
Monogalactosyl diglyceride	148	80	36	93
Digalactosyl diglyceride	106	30	20	63
Phospholipid	34	20	22	50
Sulphoquinovosyl diglyceride	10	10	10	10

1. Wintermans (1960)
2. Allen, Hirayama and Good (1966)
3. Roughan and Batt (1969)

The photosynthetic tissues (Roughan and Batt, 1969) represent the average of twenty species ranging from green algae to leaves of higher plants. The sulpholipid value was taken as 10 in each case.

fold in the morning as compared with later in the day. The wax beans in this case were harvested immediately after the plants had been in darkness for 12 hours. This may be the reason for the lower sulpholipid concentration in the wax bean chloroplasts as compared to the other studies. The species, age of the plant and the conditions of growth may also affect the ratios. The wax bean chloroplasts were harvested after 12 days and were grown under artifical light, whereas the spinach chloroplasts (Wintermans, 1960) and the spinach lamellae (Allen, Hirayama and Good, 1966) were from older plants which were grown in the field. The ratios of the lipids in the photosynthetic tissues are an average of twenty different tissues, ranging fram green alga to the leaves of higher plants (Roughan and Batt, 1969). The phospholipids are higher in this case because of the contribution from other organells as well as chloroplasts.

Other than the lower sulpholipid concentration the glycerolipids of the wax bean chloroplasts follow the same trend as those of spinach chloroplasts, spinach lamellae and other photosynthetic tissues.

Fatty Acids

Gas liquid chromatography of the fatty acids from monogalactosyl diglyceride, digalactosyl diglyceride and sulpholipid revealed the presence of major amounts of linolenic acid (18:3), high amounts of palmitic acid (16:0) and minor amounts of lauric acid (12:0), myristic acid (14:0), palmitoleic acid (16:1), palmitolenic acid (16:2), stearic acid (18:0), oleic acid (18:1), and linoleic acid (18:2).

The results for the individual glycolipids are given in Fig. 11 and Table IV, and are expressed as fatty acids in per cent of total peak area of components being detected. It may be seen that linolenic acid is by far the most abundant fatty acid in the chloroplast. In monogalactosyl diglyceride it is almost the exclusive fatty acid. It represented more than 75% of the fatty acids of digalactosyl diglyceride, sharing the glyceride mainly with palmitic acid. In the sulpholipids less than half of the fatty acid was linolenic acid with the remainder being predominantly palmitic acid.

The fatty acid composition of monogalactosyl diglyceride, digalactosyl diglyceride and sulphoquinovosyl diglyceride were slightly different from those reported previously from spinach chloroplast lamellae (Allen, Good, Davis, Chisum and Fowler, 1966) and spinach chloroplasts (Allen, Good, Davis and Fowler, 1964) which were similar in fatty acid composition. They reported that the monogalactosyl diglyceride in spinach lamellae contained 71.9 per cent linolenic acid as compared to 91.6 per cent found in the wax bean chloroplast in this study. Digalactosyl diglyceride from the spinach lamellae contained 86.7 per cent as compared to 75.3 per cent in the wax bean chloroplast. The 16:3 fatty acid of the monogalactosyl diglyceride and digalactosyl diglyceride represented 25 and 5 per cent respectively in the spinach lamellae, but there were only trace amounts in the bean chloroplast. On the other hand, palmitic acid was found only as trace amounts in these lipids of the spinach lamellae but made up 15 per cent of the fatty acids of digalactosyl diglyceride from bean chloroplasts. The fatty acid

Fig. 11. Chromatograms of Fatty Acids of the Glycolipids Extracted from Bean Chloroplasts. An Aerograph model 200 equiped with a hydrogen flame ionization detector was used. Approximately 5 ul of the methylated fatty acids from the diglycerides was injected into a 10 foot by 1/8 inch stainless steel column packed with diethylene glycol succinate (20%) on 60-80 mesh chromosorb P. The column temperature was 195°C, injector temperature 240°C, and the flow rate of nitrogen was 25 ml per min. By matching the fatty acids with known standards the peaks were identified as (1) methyl laurat; (2) methyl myristate; (3) methyl palmitate; (4) methyl palmitoleate; (5) methyl palmitolenate; (6) methyl stearate; (7) methyl oleate; (8) methyl linoleate and (9) methyl linolenate.

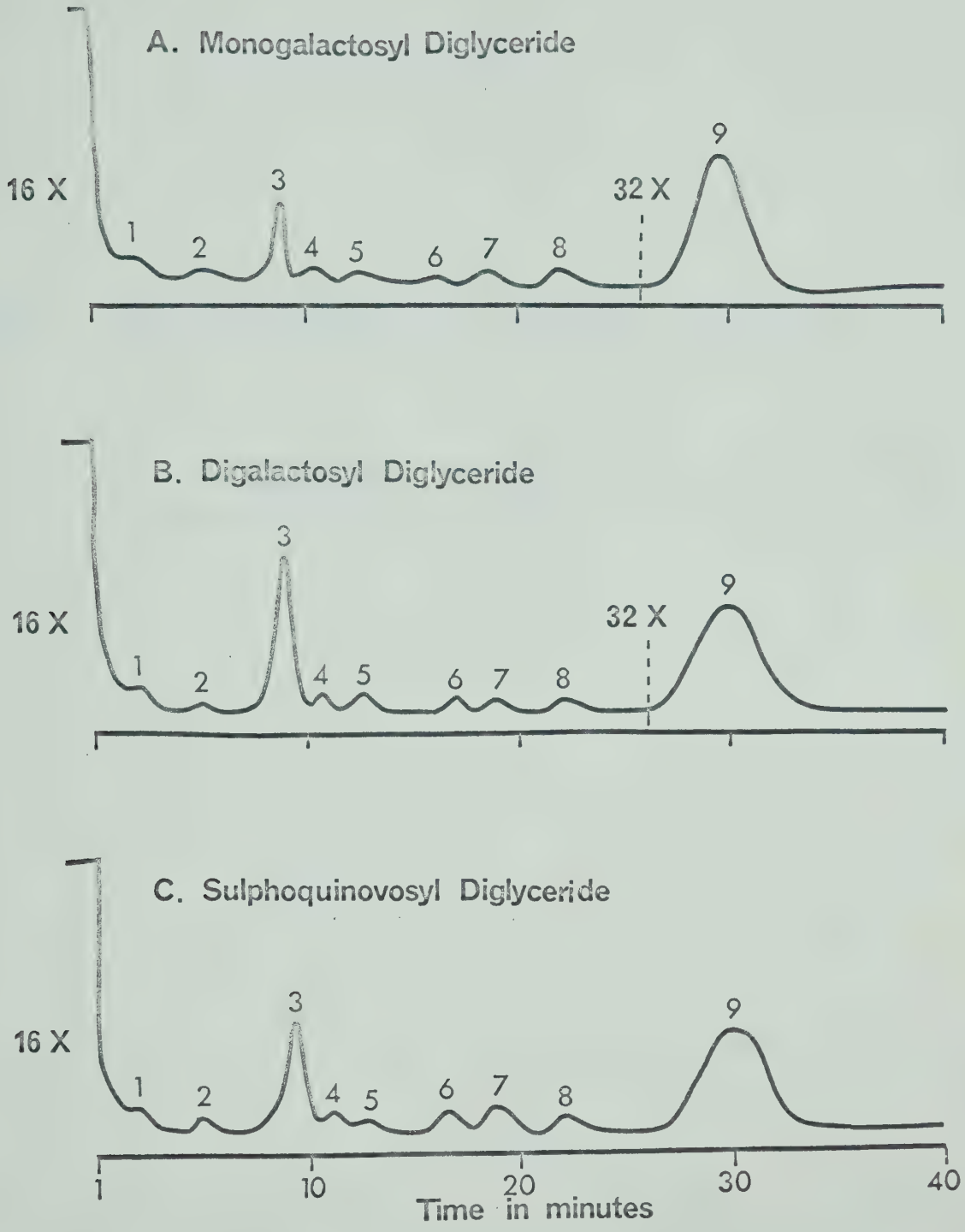
A. Monogalactosyl Diglyceride
16 X
32 X
1
2
3
4
5
6
7
8
9
B. Digalactosyl Diglyceride
16 X
32 X
1
2
3
4
5
6
7
8
9
C. Sulphoquinovosyl Diglyceride
16 X
1
2
3
4
5
6
7
8
9
1
10
20
30
40
Time in minutes

Figure 11

Table IV. Fatty Acid Composition (% peak area) of the Glycolipids from the Chloroplasts of Kinghorn Wax Bean Leaves

Fatty Acid	Monogalactosyl diglyceride	Digalactosyl diglyceride	Sulphoquinovosyl diglyceride
12:0	0.6	1.2	3.3
14:0	0.4	0.6	4.9
16:0	3.3	15.1	21.3
16:1	0.3	0.7	4.9
16:2	0.1	0.9	3.3
18:0	1.3	3.1	6.6
18:1	0.9	1.7	8.2
18:2	1.5	1.4	4.9
18:3	91.6	75.3	42.6

composition of sulphoquinovosyl diglyceride from the wax bean chloroplasts was however, similar to this component from spinach lamellae.

Sastry and Kates (1963) reported that monogalactosyl diglyceride from runner-bean leaves contained 96 per cent of 18:3 and O'Brien and Benson (1964) reported it to be 95 per cent in alfalfa leaves. Digalactosyl diglyceride from runner-bean leaves and alfalfa leaves contained 93 and 82 per cent of 18:3 respectively, the remaining acids being 16:0 in each case. No 16:3 fatty acid was reported in either case. The fatty acid composition of the sulpholipid from alfalfa leaves also resembled very closely that of the wax bean chloroplasts.

Monogalactosyl diglyceride and digalactosyl diglyceride were found to resemble each other closely in fatty acid composition. These findings may be used to support the hypothesis that monogalactosyl diglyceride is converted to digalactosyl diglyceride by galactosylation. Sulphoquinovosyl diglyceride, on the other hand, is much more saturated and is more likely to have arisen from an independent diglyceride pool.

A definite conclusion can be drawn that the lipids of the chloroplast are highly unsaturated. It is surprising that lipids with such a high degree of unsaturation are present at the site involved in light absorption and oxygen evolution, both conditions which favor oxidation of unsaturated fatty acids. Very little oxidized fatty acids were found in this study, and those that were detected may have arisen in the course of extraction and sample preparation. Therefore it seems that the unsaturated fatty acids

are protected from oxidation in the chloroplast by the presence of antioxidants or by their inaccessibility within the chloroplast lipoprotein.

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APPENDIX

Table i. Buffer for Extraction of Chloroplasts. This makes two litres of 0.33 M sucrose phosphate buffer.

Sucrose	225.6 grams
KCl	1.492 grams
$MgCl_2 \cdot 6H_2O$	2.020 grams
KH_2PO_4	27.20 grams
K_2HPO_4	34.80 grams
pH	6.80

Table ii. Phosphate Buffer. This is brought to a final volume of one litre.

KH_2PO_4	13.60 grams
K_2HPO_4	17.40 grams
pH	6.80

Table iii. Density Gradient Buffers. All quantities are for 500 ml of solution.

1.0 M (a)	Sucrose	171.2 grams
1.5 M (b)	Sucrose	256.8 grams
2.0 M (c)	Sucrose	342.4 grams
	KCl	0.373 grams
	$MgCl_2.6H_2O$	0.505 grams
	KH_2PO_4	0.680 grams
	K_2HPO_4	0.870 grams
	pH	6.80